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(54) Title: CHEMILUMINESCENT HEMOGLOBIN ASSAY

(57) Abstract

The hemoglobin content of a sample is determined by chemiluminescence, based on the ability of hemoglobin to absorb radiation emitted by a chemiluminescent reaction. To perform the assay, the sample is placed in a liquid medium with a compound that is susceptible to a chemiluminescent reaction, and the medium is exposed to conditions causing the chemiluminescent reaction to occur. The amount of emission created by the reaction and not absorbed by the hemoglobin is then detected and compared to calibrators or standards as a measure of the amount of hemoglobin present. By combining the above with procedures that separate and quantify glycosylated hemoglobin, the assay can be used to determine glycosylated hemoglobin as a proportion of the total.

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CHEMILUMINESCENT HEMOGLOBIN ASSAY

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention lies in the chemistry of detecting or analyzing for hemoglobin or hemoglobin fractions. Assays in accordance with this invention are useful in the diagnosis of certain disease conditions and in the monitoring of a patient undergoing treatment for such disease conditions.

2. Description of the Prior Art

Abnormal levels of hemoglobin, either high or low, in various bodily fluids are recognized indicators of a variety of abnormal physiological conditions. Hemoglobinemia, or the presence of free hemoglobin in the blood plasma, for example, is an indicator of intravascular hemolysis. Other examples are hemoglobinocholia, which is the presence of hemoglobin in the bile, and hemoglobinuria, which is the presence of hemoglobin in the urine. The detection of abnormal levels of hemoglobin in these bodily fluids is associated with a variety of abnormalities.

A portion of the hemoglobin in mammalian blood is normally glycosylated, and the amount and/or proportion of glycosylated hemoglobin relative to total hemoglobin is an indicator of the presence or absence of diabetes mellitus. In known diabetics, monitoring of the level of glycosylated hemoglobin is a useful means of determining the degree of glucose intolerance and of monitoring the effectiveness of the therapeutic methods used to manage the condition.

Various methods are known in the art for determining the level of glycosylated hemoglobin. Prominent among these are ion-exchange chromatography, thiobarbituric acid methods, isoelectric focusing, immunoassay, and affinity chromatography.

Chromatographic separations have inherent limitations of equilibration and elution times, size limitations of the column or separation medium, difficulties in calibrating the results, and the need to maintain high resolution, and few of these methods are susceptible to automation for the uniform and reproducible handling of a large number of samples.

The disease conditions referred to above are associated with abnormal levels of normal (non-mutated) hemoglobin. Other hemoglobin-associated diseases arise from hemoglobin mutations. Examples of these are α-thalassemia, β-thalassemia, and single-point mutated hemoglobins such as hemoglobin S (sickle-cell anemia), C, D, E and O. These mutations further detract from the ability to obtain accurate determinations of glycosylated hemoglobin by some of the methods listed in the previous paragraph.

Clinicians would therefore benefit from hemoglobin assays that can provide accurate determinations of both total hemoglobin and percent glycosylated hemoglobin in non-mutated form as well as hemoglobin that has been altered by genetic mutations in the hemoglobin genes. The procedures would also benefit from simplified and reproducible detection methods that are susceptible to automation.

SUMMARY OF THE INVENTION

It has now been discovered that hemoglobin can be detected and quantified by a light absorption assay, based on the ability of hemoglobin to absorb radiation energy emitted by a chemiluminescent reaction. According to this invention, a sample suspected of containing hemoglobin, or a sample known to contain hemoglobin but of an unknown amount, is combined in a liquid medium with a species that is capable of undergoing a chemiluminescent reaction. The liquid medium is then exposed to conditions that cause the chemiluminescent reaction to occur, and a measurement is taken of the net radiation representing the radiation emitted by the reaction less that absorbed by hemoglobin in the medium. The compound undergoing the chemiluminescent reaction will be selected such that the wavelength range of the radiation emitted by the reaction and the wavelength range of absorption by hemoglobin define a region of overlap. Detection is performed at a wavelength within that region of overlap.

To distinguish between different fractions of hemoglobin in a single sample, the fractions are separated by chemical or physical means before the chemiluminescent reaction is induced. One means of achieving separation is by a selective binding reaction between one of the fractions in the liquid sample and a binding agent bonded to the surface of a solid phase, followed by separation of the solid and liquid phases from each other. When the fraction of interest is the non-glycosylated fraction or any other fraction whose concentration is high enough to absorb a measurable amount of chemiluminescent

emission, quantitation can be achieved by the same method as described above for total hemoglobin, *i.e.*, as an inverse relation based on absorption. When the fraction of interest is a glycosylated fraction or any other fraction whose concentration is too low to absorb a measurable amount of chemiluminescent emission, quantitation can be achieved in a direct relation by a binding reaction between the fraction and the chemiluminescent compound. Details of these and other procedures in accordance with this invention are provided below.

The assays of this invention are well suited to automation, and can be performed in equipment currently available from suppliers of analytical instrumentation.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

A wide range of compounds susceptible to chemiluminescent reactions are useful in the practice of the present invention. The compounds of particular interest are ones that undergo the reaction upon a change of chemical environment, such as the addition of a further reagent or a change in pH. Examples of classes of chemiluminescent compounds meeting this description are oxalates, pyridazine-1,4-diones, and acridinium compounds. The oxalates include esters, amides and sulfonamides of oxalic acid, particularly electronegatively substituted aromatic and aliphatic esters, amides and sulfonamides. Other useful oxalates are O-oxalyl hydroxylamine derivatives, oxalcarboxylic anhydrides, and oxalyl halides. Examples of specific oxalates are bis(1-1-H)-2-pyridonyl)glyoxal, bis(2,6-difluorophenyl)oxalate, and bis(4-nitro-2-(3,6,9-trioxadecylcarbonyl)phenyl)oxalate. The pyridazine-1,4-diones include luminol (3-aminophthalhydrazide), isoluminol (4-amino-phthalhydrazide), naphthyl-1,2-pyridazine, benzoperilenyl-1,12-pyridazine, 2-phenyl-3-hydroxypyrido-3,4-pyridazine, and further analogs bearing the phthalazine-1,4-dione group, the pyridopyridazine group, or other cyclic or aromatic 1,4-diones. The acridinium compounds include 10,10'-dimethyl-9,9'-biacridan, lucigenin (bis-N-methylacridinium nitrate), 9-acridinecarboxylic acid (and the acid halide and phenyl ester thereof), 3,6-bis-(dimethylamino)acridine (and halide, nitrate and sulfate salts thereof), 3,6-bis(diamino)-acridine (and halide, nitrate and sulfate salts thereof), 3,6-bis(diamino)-N-methylacridinium chloride, 2,7-bis(dimethyl)-3,6-bis(diamino)acridine (and halide, nitrate and sulfate salts thereof), and further analogs bearing the acridine group.

Initiation of the chemiluminescent reaction is achieved by exposing the compound to the appropriate reactant or reaction condition, which will vary depending on the particular compound chosen. The reaction can thus be initiated by the addition of a reactant that will combine with the compound in the chemiluminescent reaction or in the

first reaction of a reaction sequence or cascade that includes a chemiluminescent reaction. The chemiluminescent reaction can likewise be initiated by the addition of a catalyst that catalyzes the chemiluminescent reaction or initiates a reaction sequence or cascade that includes a chemiluminescent reaction. Alternatively, the chemiluminescent reaction can be initiated by a change in pH to a value within a range in which the reaction will occur (or in which a sequence or cascade including the reaction will be initiated). When the chemiluminescent reaction requires the addition of two or more reactants or both a reactant and a change in condition, the liquid medium prior to the reaction can contain all but one of the reactants or conditions and initiation of the reaction can be achieved by adding the final reactant or converting to the final condition at the chosen time. For many chemiluminescent reactions, the added reactant is hydrogen peroxide, an acid or base, or a combination of hydrogen peroxide and an acid or base. In general, the reactants and reaction conditions needed to initiate the chemiluminescent reaction will be known to those skilled in the art, since the reactions themselves are known.

Acridinium compounds are preferred sources of chemiluminescence, since the emission spectra of these compounds overlap well with the absorption spectrum of hemoglobin. Detection will be performed at a selected wavelength that will fall within the region of overlap. The absorption spectrum of hemoglobin in base is a broad curve, with a peak at approximately 393 nm, an absorption at 370 nm of about 90% of the peak value, an absorption at 410 nm of about 81% of the peak value, an absorption at 430 nm of about 42% of the peak value, and an absorption at 450 nm of about 21% of the peak value. When acridinium compounds are used for the chemiluminescent reaction, the detection wavelength is preferably within the range of about 400 nm to about 450 nm, more preferably about 420 nm to about 440 nm, and most preferably a wavelength of about 430 nm. Initiation of the chemiluminescent reaction of an acridinium compound is preferably achieved by the addition of hydrogen peroxide and an acid followed by the addition of an alkali, preferably an inorganic base.

The term "liquid medium" is used herein to represent homogeneous liquid solutions, heterogeneous liquid mixtures such as oil-in-water and water-in-oil emulsions, and liquid-solid mixtures such as suspensions or dispersions. The compound that will undergo the chemiluminescent reaction can either be a solute in a liquid solution or one liquid phase of an emulsion. Alternatively, the compound can be immobilized on a solid phase, either by covalent bonding, affinity-type binding, immunological binding, or simple electrostatic attraction. As a further alternative, the compound can be conjugated to an affinity-type or immunology-type binding member, which can in turn be affixed or otherwise immobilized on a solid support or suspended in solution.

The invention is useful for the detection of hemoglobin or the percentage of any of various hemoglobin fractions in samples from a variety of sources and in a variety of

physical forms. The sample can itself be a liquid, or any substance dissolved or dispersed in a liquid. Biological samples include blood, sera, urine, and other bodily fluids.

Mammalian blood samples are preferably treated to lyse the red blood cells prior to the analysis. Lysis of the red blood cells can be accomplished by applying a hemolysis technique to the entire blood sample or to any portion thereof that contains all or substantially all of the red blood cells. If desired, the red blood cells can be separated from whole blood by centrifugation prior to lysis.

Any conventional lysis technique can be used. One technique involves the addition of a detergent to the sample. Typical detergents are cetyl trimethyl ammonium bromide, sodium dodecyl sulfate, sodium deoxycholate, saponin, and polyoxyethylenes such as TRITON® X-100. Others will be readily apparent to those skilled in the art, as will the procedures for the most effective use of these materials. Lysis can also be achieved by ultrasonication or by the use of a hypotonic solution.

In assays requiring the separation of one hemoglobin fraction from the remainder of the hemoglobin present, the separation can likewise be achieved by methods known in the art. To separate glycosylated hemoglobin from nonglycosylated hemoglobin, for example, a particularly effective and convenient method is the selective reaction of glycosylated hemoglobin in the liquid medium with a dihydroxyboryl compound immobilized on a solid support, followed by separation of the solid and liquid phases. Examples of dihydroxyboryl compounds that will serve this purpose are alkaneboronic acids, phenylboronic acid, alkylphenylboronic acids, nitrophenylboronic acids, and aminophenylboronic acids. Phenylboronic acids are particularly preferred. The dihydroxyboryl compound can be immobilized on the support by covalent bonding or other means of affixation, and the support can be any solid phase that will not otherwise react with any component of the liquid medium or dissolve in the liquid medium.

Certain hemoglobin fractions are too low in concentration (once the necessary dilutions of the sample have been performed) to cause a detectable change in the chemiluminescent emission. Hemoglobin A_{1c} is one such fraction. Absorption of the chemiluminescent emission is thus an impractical basis for the assay once the fraction has been isolated from the remainder of the hemoglobin. For such fractions, alternative methods such as those involving a direct quantitation can be used. Direct quantitation can be performed in any of various ways that will be readily apparent to those skilled in the art, and the result can then be combined with an assay for total hemoglobin by absorption of chemiluminescence in accordance with this invention, to express the fraction as a percentage.

One direct quantitation method that is particularly convenient in the context of this invention is a method involving the same chemiluminescent reaction used in the absorption assay. According to this method, the fraction of interest, once isolated, is joined to the

compound that undergoes the chemiluminescent reaction, the two forming a conjugate in which the hemoglobin molecule and the chemiluminescent compound are present in a 1:1 molar ratio, or any other fixed integral molar ratio depending on the means of conjugation. Excess (unconjugated) compound is then removed, and the chemiluminescent reaction is permitted to occur, the resulting emission being directly proportional to the amount of conjugate (and hence the amount of the hemoglobin fraction) present. Absorption is not determined in this assay, since at the molar ratios present in conjugates, the amount of absorption by the hemoglobin moiety is either too small to be detected, or is compensated for by appropriate calibration of the assay.

Automation of the assay, either for total hemoglobin or a hemoglobin fraction, is readily achieved by the use of instrumentation currently available and familiar to those skilled in the art. Automation permits the assay of a multitude of samples, and affords a high degree of control over assay conditions, sequences and procedures, reproducibility among different samples, and automated determination of results.

One example of an automated instrument on which the assays can be performed is the Automated Chemiluminescence System ACS:180, Ciba-Corning Diagnostics Corp., E. Walpole, Massachusetts, USA. This instrument is designed to perform up to 180 assays per hour with computer-controlled timing and sequences, and includes a cuvette loader/process track, a sample handling system, a reagent handling system, a separation and washing system, a luminometer, and a data collection and processing system. Although designed for immunoassays, the ACS:180 can be used in the present invention, which does not strictly rely on immunological reactions, and for which certain embodiments do not involve immunological binding at all. The ACS:180 operates by measurements of chemiluminescence from an acridinium ester used as a tracer, contains a 60-position tray for loading patient samples, calibrators, controls and diluents, and handles sample volumes ranging from 10 to 200 μL. Reagents include a first reagent consisting of 0.5% hydrogen peroxide in 0.1 M nitric acid, and a second reagent consisting of 0.25 M sodium hydroxide, both in aqueous solutions, and detection is performed by a photo-multiplier tube. As a solid phase, the instrument uses micron-sized paramagnetic particles whose ferric oxide coating enables them to be chemically derivatized to bind to a fraction of the sample and thereby permit separation of the fraction by separation of the remaining liquid from the particles, or vice versa. Derivatizable paramagnetic particles can be used as a support for the binding of the acridinium ester, for the binding of a dihydroxyboryl compound when one is used, or for other types of binding as required by the assay.

When paramagnetic particles are used, their paramagnetic character assists in the separation of the liquid phase from the solid phase by permitting the particles to be retained in the sample cuvettes as the liquid is aspirated and the cuvette subsequently washed. Retention of the particles is achieved by imposing a magnetic field on the cuvette

so that the particles, that are otherwise suspended in the liquid medium residing in the cuvette, are immobilized the particles against the cuvette wall. Particles of any paramagnetic material can be used; suitable materials will be readily apparent to those skilled in the art.

The following examples are offered for purposes of illustration, and are not intended to impose limits on the scope of the invention.

EXAMPLE 1

A procedure for the determination of total hemoglobin in whole blood in accordance with this invention is as follows.

Whole blood is diluted into a buffer solution at a dilution of from about 1:200 to about 1:500, and dispensed into a reaction cuvette. An aliquot of an aqueous solution of lucigenin is added to the cuvette. While the amount of total hemoglobin in the whole blood sample is not known, a range within which the amount is expected to reside will generally be known, and the quantity of lucigenin in the aliquot added will be sufficient that the chemiluminescent radiation emitted by the lucigenin upon activation will be more than the amount that can be absorbed by a hemoglobin quantity at the upper end of the range of expectation. Thus, less than all of the emitted radiation will be absorbed by the hemoglobin.

Once the lucigenin is added, a solution of hydrogen peroxide (0.5% by weight) and nitric acid (0.1 M) is added. The cuvette is then aligned with a photomultiplier detector and an aqueous solution of 0.25 M sodium hydroxide is added. The intensity of the resulting emission is measured for about 5 seconds. An adequate number of calibrator solutions is used to calibrate the assay, and thereby to accurately correlate the emission intensity (unabsorbed by the hemoglobin) with the amount of hemoglobin in the sample.

EXAMPLE 2

The following is a procedure for the determination of glycosylated hemoglobin as a percentage of the hemoglobin content in a sample of whole blood.

A sample of whole blood is lysed according to any of the procedures described above. The lysed sample is then diluted into a buffer solution at a dilution of from about 1:200 to about 1:500, and placed in a reaction cuvette to which ferric oxide particles with phenylboronate groups covalently linked thereto are also added. The mixture of liquid and

particles is then incubated at room temperature for at least about ten minutes. The cuvette is then placed in a magnetic field, causing the particles to adhere to one side of the cuvette. Liquid is then aspirated from the cuvette, and the cuvette and particles, still under the influence of the magnetic field, are washed with an appropriate wash solution.

A conjugate of lucigenin and anti-human hemoglobin is then added to the cuvette, in excess amount. The anti-human hemoglobin portion of the conjugate is one that exhibits specific binding to either the alpha- or the beta-subunit of hemoglobin. Once combined in the cuvette, the conjugate and particles are incubated for a sufficient period of time to permit a full immunological reaction resulting in the binding of the lucigenin to the particle-immobilized glycosylated hemoglobin. A magnetic field is once again imposed, causing the particles to adhere to one side of the cuvette. Liquid is then aspirated as before, and the particles are washed with an appropriate wash solution.

A solution of hydrogen peroxide (0.5% by weight) and nitric acid (0.1 M) is then added as in Example 1. The cuvette is then aligned with a photomultiplier detector and an aqueous solution of 0.25 M sodium hydroxide is added. The intensity of the resulting emission is measured for about 5 seconds. An adequate number of calibrator solutions is used to calibrate the assay, and thereby to accurately correlate the emission intensity (representing the amount of lucigenin retained by the glycosylated hemoglobin on the particles) with the amount of glycosylated hemoglobin in the sample. Note that the amount of emission absorbed by the glycosylated hemoglobin, if any, is negligible due to the small quantity of glycosylated hemoglobin present. The measured emission is thus directly proportional to the amount of lucigenin remaining in the cuvette, which in turn is directly proportional (*i.e.*, in a molar ratio) to the amount of glycosylated hemoglobin present.

To obtain the percentage of glycosylated hemoglobin relative to total hemoglobin, one performs a separate analysis according to Example 1, and then compares the two results.

The foregoing is offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that the operating conditions, materials, procedural steps and other parameters of the system described herein may be further modified or substituted in various ways without departing from the spirit and scope of the invention.

WE CLAIM:

1. A method for detecting the presence of a hemoglobin compound in a liquid sample, said method comprising:

(a) forming a mixture of said sample and a compound capable of undergoing a chemiluminescent reaction, in a liquid medium, said chemiluminescent reaction being one that causes emission of radiation over a wavelength range at least a portion of which falls within an overlapping range with the absorption wavelength range of hemoglobin;

(b) subjecting said compound to conditions causing said chemiluminescent reaction to occur; and

(c) detecting radiation energy generated in said liquid medium in said overlapping range by said chemiluminescent reaction and not absorbed by said hemoglobin compound, and comparing said radiation energy thus detected to a standard as indicative of the quantity of said hemoglobin compound in said sample.

2. A method in accordance with claim 1 in which said compound capable of undergoing a chemiluminescent reaction is a member selected from the group consisting of oxalates, pyridazine-1,4-diones, and acridinium compounds.

3. A method in accordance with claim 1 in which said compound capable of undergoing a chemiluminescent reaction is an acridinium compound and (b) comprises adding peroxide and alkali to said liquid medium.

4. A method in accordance with claim 1 in which said compound capable of undergoing a chemiluminescent reaction is an acridinium compound and (b) comprises adding hydrogen peroxide and nitric acid to said liquid medium, followed by adding an inorganic base to said liquid medium.

5. A method for determining the amount of glycosylated hemoglobin in a liquid sample containing both glycosylated and non-glycosylated hemoglobin, said method comprising:

(a) contacting said sample with sufficient dihydroxyboryl compound immobilized on a solid phase to bind thereto substantially all glycosylated hemoglobin in said liquid sample;

(b) separating said solid phase from unbound species remaining in said liquid sample;

(c) further binding to said glycosylated hemoglobin a compound capable of undergoing a chemiluminescent reaction;

(d) subjecting said compound thus bound to glycosylated hemoglobin to conditions causing said chemiluminescent reaction to occur; and

(e) detecting radiation energy generated by said chemiluminescent reaction from said compound thus bound to glycosylated hemoglobin and comparing said radiation energy thus detected to a calibrator as indicative of the quantity of glycosylated hemoglobin in said sample.

6. A method in accordance with claim **5** in which said dihydroxyboryl compound is a member selected from the group consisting of alkaneboronic acids, phenylboronic acid, alkylphenylboronic acids, nitrophenylboronic acids, and aminophenylboronic acids.

7. A method in accordance with claim **5** in which said solid phase is comprised of a plurality of solid particles insoluble in said liquid medium.

8. A method in accordance with claim **7** in which said solid particles are paramagnetic, and (b) comprises magnetically separating said solid particles from said liquid medium.

9. A method for determining the proportion of glycosylated hemoglobin relative to total hemoglobin in a liquid sample containing both glycosylated and non-glycosylated hemoglobin, said method comprising:

(a) contacting a first portion of said sample with sufficient dihydroxyboryl compound immobilized on a solid phase to bind thereto substantially all glycosylated hemoglobin in said liquid sample;

(b) separating said solid phase from unbound species remaining in said liquid sample;

(c) further binding to said glycosylated hemoglobin a compound capable of undergoing a chemiluminescent reaction;

(d) subjecting said compound thus bound to glycosylated hemoglobin to conditions causing said chemiluminescent reaction to occur;

(e) detecting radiation energy generated by said chemiluminescent reaction from said compound thus bound to glycosylated hemoglobin and comparing said radiation energy thus detected to calibrators as indicative of the quantity of glycosylated hemoglobin in said sample;

(f) forming a mixture of a second portion of said sample and a second compound capable of undergoing a chemiluminescent reaction, in a liquid medium, said chemiluminescent reaction being one that causes emission of radiation over a wavelength range at least a portion of which falls within an overlapping range with the absorption wavelength range of hemoglobin;

(g) subjecting said second compound to conditions causing said chemiluminescent reaction to occur;

(h) detecting radiation energy generated in said liquid medium in said overlapping range by said chemiluminescent reaction and not absorbed by said hemoglobin compound, and comparing said radiation energy thus detected to calibrators as indicative of the quantity of total hemoglobin compound in said sample; and

(i) comparing the result obtained in step (e) to the result obtained in step (h) to determine the proportion of glycosylated hemoglobin relative to total hemoglobin in said sample.

10. A method in accordance with claim **9** in which said first compound capable of undergoing a chemiluminescent reaction and said second compound capable of undergoing a chemiluminescent reaction are identical.

INTERNATIONAL SEARCH REPORT

Im tional Application No
PCT/US 98/09867

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/72 G01N33/543 G01N33/551

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 9436 Derwent Publications Ltd., London, GB; AN 94-290022 XP002073187 NAKANO KIYOKAZO: "Simultaneously determining special component in blood and haemoglobin - by contacting oxidase with blood serum or plasma to form exciting agent, adding e.g. luminol and catalyst and then measuring" & JP 06 217 794 A (SHIMADZU CORP) , 9 August 1994	1,2
Y	see abstract --- -/--	3,4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 July 1998	20/08/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Im tional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 9252 Derwent Publications Ltd., London, GB; AN 92-428422 XP002073188 FUJII TAKASHI ET AL.: "Determining human haemoglobin in excrement for diagnosing intestinal cancer - by chemiluminescent method in presence of oxidising agent, using anti-human haemoglobin antibody and luminol" & JP 04 324 358 A (TEIKOKU SEIYAKU KK) , 13 November 1992	1
Y	see abstract	5-10
X	PATENT ABSTRACTS OF JAPAN vol. 095, no. 009, 31 October 1995 & JP 07 140143 A (INAX CORP), 2 June 1995	1
Y	see abstract	5-10
P,X	WO 97 33884 A (SS PHARMACEUTICAL CO ;IMAI KAZUHIRO (JP); ETO HIROMICHI (JP); KOTS) 18 September 1997 see page 26 - page 31; examples 23,24	5-10
E	& EP 0 844 246 A (SS PHARMACEUTICAL CO) 27 May 1998 see page 17, line 1 - page 18, line 25; examples 23,24	5-10
Y	EP 0 330 433 A (GEN PROBE INC) 30 August 1989 see page 2 - page 3 see page 7, line 35 - line 36; example 1	3,4
Y	EP 0 761 652 A (MOCHIDA PHARM CO LTD) 12 March 1997 see page 28, line 47 - line 48; example 28.3 see page 29, line 5 - line 7; example 28.4	4
Y	WO 93 18407 A (ABBOTT LAB) 16 September 1993 see the whole document	5-10

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	D C KLENK, G T HERMANSON, R I KROHN, E K FUJIMOTO, A KRISHNA MALLIA, P K SMITH, J D ENGLAND, H-M WIEDMEYER, R R LITTLE ET AL.: "Determination of Glycosylated Hemoglobin by Affinity Chromatography: Comparison with Colorimetric and Ion-Exchange Methods, and Effects of Common Interferences" CLINICAL CHEMISTRY, vol. 28, no. 10, 1982, pages 2088-2094, XP002073184 see the whole document	5-10
Y	EP 0 708 337 A (FUJI PHOTO FILM CO LTD) 24 April 1996 see the whole document	5-10
Y	EP 0 573 972 A (FUJIREBIO KK) 15 December 1993 see example 4	5-10
A	GB 2 063 469 A (FISHER SCIENTIFIC CO) 3 June 1981 see the whole document	1-10
A	EWETZ L ET AL: "FACTORS AFFECTING THE SPECIFICITY OF THE LUMINOL REACTION WITH HEMATIN COMPOUNDS" ANALYTICAL BIOCHEMISTRY, vol. 71, no. 2, 1976, pages 564-570, XP000618259 see the whole document	1-10
A	I WEEKS, A K CAMPBELL, J S WOODHEAD: "Two-Site Immunochemiluminometric Assay for Human alpha1-Fetoprotein" CLINICAL CHEMISTRY, vol. 29, no. 8, 1983, pages 1480-1483, XP002073185 see the whole document	5-10
A	A P RICHARDSON, J B KIM, G J BARNARD, W P COLLINS, F MCCAPRA: "Chemiluminescence Immunoassay of Plasma Progesterone, with Progesterone-Acridinium Ester Used As the Labelled Antigen" CLINICAL CHEMISTRY, vol. 31, no. 10, 1985, pages 1664-1668, XP002073186 see the whole document	5-10

INTERNATIONAL SEARCH REPORT

information on patent family members

Int ional Application No
PCT/US 98/09867

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9733884 A	18-09-1997	JP 9249662 A	22-09-1997
		JP 9249663 A	22-09-1997
		EP 0844246 A	27-05-1998
EP 0330433 A	30-08-1989	US 4950613 A	21-08-1990
		AT 137021 T	15-05-1996
		AU 626319 B	30-07-1992
		AU 4072389 A	22-09-1989
		CA 1333396 A	06-12-1994
		DE 68926259 D	23-05-1996
		DE 68926259 T	19-09-1996
		DK 530489 A	27-12-1989
		ES 2012323 T	16-06-1996
		JP 2503268 T	11-10-1990
		PT 89834 A,B	04-10-1989
		WO 8908256 A	08-09-1989
EP 0761652 A	12-03-1997	CA 2182457 A	02-02-1997
		JP 9100415 A	15-04-1997
WO 9318407 A	16-09-1993	CA 2102417 A	05-09-1993
		EP 0589001 A	30-03-1994
		JP 6507978 T	08-09-1994
		US 5478754 A	26-12-1995
EP 0708337 A	24-04-1996	JP 8122335 A	17-05-1996
EP 0573972 A	15-12-1993	JP 2596322 B	02-04-1997
		JP 6066796 A	11-03-1994
		US 5470759 A	28-11-1995
		JP 2596321 B	02-04-1997
		JP 6066795 A	11-03-1994
GB 2063469 A	03-06-1981	CA 1135620 A	16-11-1982
		DE 3039157 A	27-08-1981
		FR 2468125 A	30-04-1981
		JP 56096249 A	04-08-1981
		US 4375972 A	08-03-1983